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INVESTIGATION OF OPERATING PARAMETERS IN HIGH-PERFORMANCE DISPLACEMENT CHROMATOGRAPHY

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SUMMARY

The effect of operational parameters of displacement chromatography was examined in the separation of various mixtures such as that of the main hydrolysis products of methylfurylbutyrolactone, a potential anticancer drug, the diastereoisomers benzoyl-D- and benzoyl-L-phenylalanyl-L-alanyl-L-proline, as well as polyethylene glycol homologues containing 1–10 ethylene oxide units. The chromatograph was assembled from modules generally used in analytical high-performance liquid chromatography (HPLC) and the column effluent was analyzed by an on-line HPLC unit at 30-sec intervals. Octadecyl-silica was used throughout as the stationary phase. Derivatives of ethylene glycol and propylene glycol as well as tetrabutylammonium bromide and *n*-butanol were used as displacers.

The throughput was used as the measure of efficiency. In the absence of axial dispersion, for a given separation various displacers are expected to yield the same efficiency if the slope of the operating line is kept the same by appropriate adjustment of displacer concentrations. In practice, however, the optimum slope of the operating line has to be determined experimentally as most available chromatographic systems depart from ideal behavior. The dependence of the throughput on the flow-rate and feed load also indicated the presence of non-equilibrium phenomena and the optimum value of these parameters was established experimentally. In most cases water was used as the carrier solvent but the separation of poorly soluble peptides required the use of hydro-organic carriers. Results obtained with octadecyl-silicas of different origin and a given displacer were found to vary significantly suggesting that even for stationary phases of the same type the selection of displacer requires special consideration. Most experiments were carried out with columns having dimensions customary in analytical HPLC. Increasing the inner diameter of the column did not result in the expected increase in throughput probably due to poor distribution of the sample at the column entrance. Therefore scaling-up the process requires careful engineering of inlet conditions. Throughput can be increased by connecting a small inner diameter column to the outlet of a large diameter preparative column.

As theoretical predictions for ideal displacement chromatography do not hold in practice when axial dispersion is significant, optimization of the process requires experimental support. The results obtained in the separation of a variety of mixtures shed light on the most important operational aspects of displacement chromato-

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graphy and suggest approaches to find optimum conditions. They also point to displacement chromatography as a powerful technique for preparative separations.

INTRODUCTION

The potential of displacement chromatography was recognized forty years ago by Tiselius[1], and the theoretical foundation of this mode of chromatography has been well established[2–6]. The technique has been eclipsed by linear elution chromatography which is characterized by retention values and peak shape that are independent of concentration and facilitates qualitative and quantitative analysis of the eluite peaks in the effluent. On the other hand displacement chromatography offers many advantages over the elution mode in preparative separations. First, the ultimate shape of the product concentration profiles is in most cases independent of the feed concentration, and therefore does not affect process efficiency. Second, the concentration of the separated products in the column effluent can be much higher than in the elution mode. Third, the speed and efficiency of the separation are easily controlled by the concentration of the displacer. Fourth, tailing is reduced as a result of self-sharpening boundaries in a well designed chromatographic system.

In the displacement mode, the column is first equilibrated with a carrier, chosen for its low retention on the stationary phase, and its ability to dissolve the feed components at sufficiently high concentrations in order to allow the introduction of large samples. In some cases the operation may require elevated column temperature in order to avoid precipitation that prevents further separation and plugs the column. Viscosity, compatibility with the detection system, toxicity and reactivity toward feed components, displacers and the wetted parts of the chromatograph are also to be considered in selecting the carrier.

After the feed mixture is introduced into the carrier stream, the solution of a suitable displacer is pumped through the column. Adsorption interferences cause the components to move down the column at speeds determined by the velocity of the displacer front. Stronger adsorbing components of the mixture displace from the surface of the stationary phase those having weaker retention until separation is achieved. The stationary phase should provide adequate selectivity and a high capacity for the components of interest as determined by their adsorption isotherms. Irreversible adsorption or catalytic activity by the stationary phase are to be avoided. The mixture separates into adjacent bands of uniform concentrations, ranked in the order of their affinity for the stationary phase, so high column efficiency is required to prevent zone intermixing.

Our work focused on the choice of the displacer, which is one of the most critical design parameters for obtaining maximum separation efficiency. It was prompted by the recognition that the current lack of theoretical and practical guides for displacer selection is perhaps the greatest impediment to succesful implementation of displacement chromatography and renewal of interest in the use of this technique for preparative chromatography. In this report certain particulars of displacer selection are pointed out and in addition other design aspects of chromatography of silica bound hydrocarbonaceous stationary phases are illustrated. In view of the great interest in purification of biological substances, the selection of mixtures used here was

aimed at demonstrating the efficiency and versatility of high-performance displacement chromatography in biochemical separations.

EXPERIMENTAL

Sample mixtures

Degradation products of MFBL. Fodor and co-workers[7,8] have recently synthesized methylfurylbutyrolactone (MFBL) (Nafocare B), which is a potential anticancer drug[9]. It readily decomposes in water and in the present study the two main hydrolytic degradation products, A and B, which have not yet been identified, were separated by displacement chromatography. MFBL for the study of the hydrolytic reaction was a gift by G. Fodor, University of West Virginia. A typical chromatogram of the mixture is shown in Fig. 1.

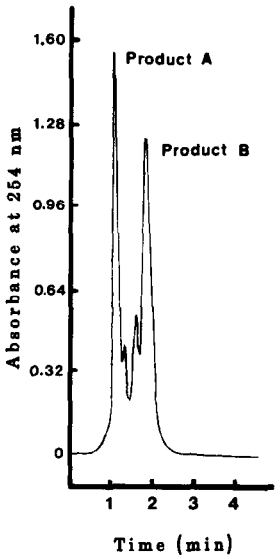
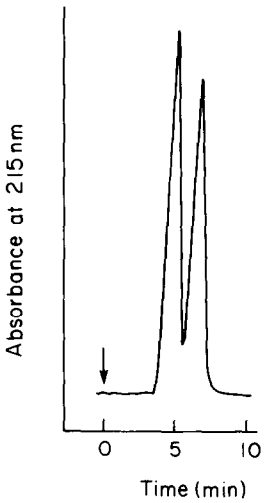


Fig. 1. Chromatogram of the MFBL degradation products. Column: 40 × 4.6 mm, 3-μm Partisil ODS. Eluent: 10 m*M* phosphate buffer, pH 2.3; flow-rate, 2 ml/min. Temperature: 22°C. Sample: 0.33 mg of the MFBL degradation products in 10 μl of water.

BPAP diastereoisomers. A mixture of the two diastereoisomeric peptides benzoyl-D- and benzoyl-L-phenylalanyl-L-alanyl-L-proline (BPAP) was kindly supplied by Dr. Bruce Pitt of the School of Medicine, Yale University. A chromatogram of the mixture is shown in Fig. 2.

Polyethylene glycol homologues. Carbowax PEG-400 was obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). It is believed to be a mixture of the first ten polyethylene glycol oligomers and its composition is illustrated by the chromatogram in Fig. 3.



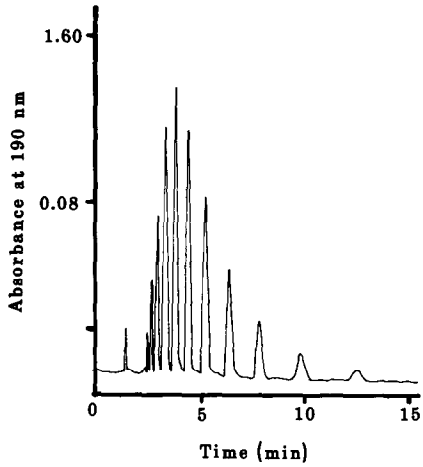


Fig. 2. Chromatogram of BPAP diastereoisomers. Column: 250 × 4.6 mm, 5-μm Supelco RP-8. Mobile phase: methanol–water (13:87); flow-rate, 1 ml/min. Temperature: 60°C. Sample: 0.4 μg of BPAP in 10 μl of water.

Fig. 3. Chromatogram of Carbowax PEG-400. Column: 250 × 4.6 mm, 5-μm Supelco RP-8. Mobile phase: acetonitrile–water (17:83); flow-rate, 1.5 ml/min. Temperature: 60°C. Sample: 0.07 μg of Carbowax PEG-400 in 10 μl of water.

Chemicals

Acetonitrile, isopropanol, *n*-butanol, methanol, sodium phosphate monobasic, phosphoric acid, Carbowax PEG-400, 600, 1000 and 4000 were obtained from Fisher Scientific. 2-(2-Butoxyethoxy)ethanol (BEE), 2-butoxyethanol (BE) and tetrabutylammonium bromide were from Aldrich (Milwaukee, WI, U.S.A.). Tripropylene glycol monomethyl ether (TPM) and dipropylene glycol monomethyl ether (DPM) were from ARCO (Philadelphia, PA, U.S.A.). Distilled water was prepared with a Barnstead distilling unit.

Apparatus and procedures

The separation of the two major degradation products of MFBL was carried out with the two chromatographs connected according to the flow sheet shown in Fig. 4. The "fractionator" chromatograph consisted of a Model 302 pump (Gilson, Middletown, WI, U.S.A.), a 250 × 4.6 mm column packed with 5-μm Zorbax ODS (DuPont) and a differential refractometer (Knauer, Berlin, F.R.G.). A Model 7010 sampling valve (Rheodyne, Berkeley, CA, U.S.A.) with a 1-ml sample loop was used to load the feed into the column. The effluent from the fractionator column was sampled every 30 sec by a Model 7413 (Rheodyne) automatic switching valve with a 10-μl sample loop and analyzed by the on-line chromatograph, *i.e.*, analyzer. It consisted of a Model 100 pump (Altex, Berkeley, CA, U.S.A.), a Model LC No. 75 spectrophotometric detector (Perkin-Elmer, Norwalk, CT, U.S.A.) and a 40 × 4.6 mm column packed with 3-μm Partisil ODS (Whatman, Clifton, NJ, U.S.A.) having a carbon content of 7.12%. The automatic sampling valve was activated through a

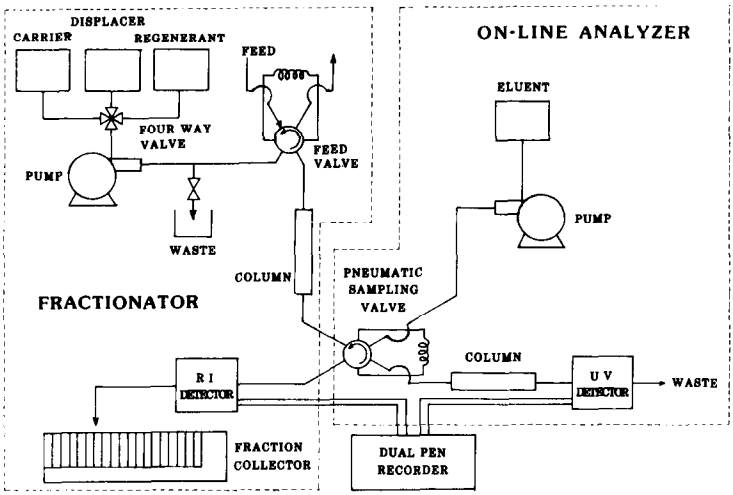


Fig. 4. Flow sheet of the combined fractionator and on-line analyzer used for the separation of the MFBL degradation products by displacement chromatography.

Model ChronTrol CD controller (Lindburg Enterprises, San Diego, CA, U.S.A.) and the detector outputs of the two chromatographs were connected to a Model BD No. 41 dual pen recorder (Kipp and Zonen, Delft, The Netherlands).

At the start of a run the column of the fractionator was first equilibrated with water, then the drain was opened and the pump purged with the displacer solution. The feed loop was filled with the mixture and the valve was turned to the "inject" position. The drain was then closed, causing the displacer to push the feed into the column. Flow-rate was 0.1 ml/min in order to be close to the ideal equilibrium condition, except in the experiments where the effect of flow-rate was studied. Each drop (35 μl) of the effluent coming from the fractionator column was collected for post-run analysis. After the emergence of the displacer front the column was regenerated by pumping isopropanol, followed by methanol through the column at a flow-rate of 1 ml/min, then the column was reequilibrated with water at a flow-rate of 1 ml/min.

The eluent for the on-line analyzer system was a solution of 6% (v/v) acetonitrile in a 10 m*M* phosphate buffer, pH 2.3. The flow-rate was 3 ml/min and the effluent was monitored at 254 nm. All the experiments were performed at a temperature of 22°C.

The chromatographic system used for the separation of BPAPs and polyethylene glycols was identical to that used in a previous study[10]. Fractions of the effluent were collected by a Model 7000 fraction collector (LKB Instruments, Rockville, MD, U.S.A.) and then analyzed after completion of the displacement chromatographic run. A Model 601 (Perkin-Elmer) dual pump system was plumbed with a system of valves to change from the carrier to the displacer stream without any change in pressure. For the separation of the BPAP diastereoisomers several columns were used: a 400 × 4.6 mm, 5-μm Spherisorb ODS, a 250 × 4.6 mm, 10-μm Partisil ODS and a 250 × 4.6 mm, 5-μm Partisil ODS column. Three 250-mm, 10-μm Partisil ODS columns having different inner diameters of 4.6 , 9.4 and 22 mm, were obtained from Whatman and used for the separation of the polyethylene glycol mixture.

Experiments were carried out with several different displacers at room temperature, or at 60°C using a Lauda WB-20/R (Brinkman) recirculating water-bath, in a wide range of conditions of flow-rate and sample size. Aliquots of the effluent fractions were diluted 25–50 fold and analyzed with a 250 × 4.6 mm, 5-μm Supelco RP-8 column, by using the same apparatus that served as on-line analyzer for the separation of the MFBL hydrolyzate and described previously. The analyses were carried out at 50–60°C using the same recirculating water-bath that was used in the displacement experiments.

The fractionator column was regenerated by purging with 20–30 column volumes of methanol, or, when an alkylammonium salt was used as the displacer, a mixture of 1% (v/v) phosphoric acid, 49% (v/v) water and 50% (v/v) isopropanol, before reequilibration with water.

Measurement of recovery and throughput

In the experiments with MFBL hydrolyzate, aliquots of the fractions of the column effluent were analyzed by using the analytical chromatograph which was otherwise employed for on-line analysis of the effluent of the displacement column. The eluent was a 10 m*M* aqueous phosphate, pH 2.3. The flow-rate was 1 ml/min, the detection wavelength was 254 nm and the column was kept at room temperature.

The recovery of a given feed component was defined for this study as the percentage of the component contained in successive effluent fractions at a purity of 100 or 96%. Throughput of a given product was evaluated by dividing the amount of recovered product by the retention time of the displacer front in the column. As displacement chromatography is preferentially carried out with two columns[11], one being regenerated while the other is used for separation, the time of regeneration was not taken into account in calculating the throughput.

RESULTS AND DISCUSSION

A major impediment to ready implementation of the displacement mode in preparative liquid chromatography is the lack of methods for selecting and optimizing the composition of the displacer solution for the separation of a given mixture after an appropriate column has been chosen.

Selection of the displacer

Basic requirements for a displacer can be deduced from both theoretical and practical considerations. The prime feature of a displacer is that its adsorption isotherm overlies the isotherms of all feed components to be displaced[12]. It should not react with any of the feed components and have sufficiently high solubility in the mobile phase. Although the displacer should adsorb strongly on the stationary phase it also has to be amenable to some scheme for ready removal when the column is regenerated[13]. Moreover, it should be easily removable from the product ahead of it that may be contaminated by the displacer. It should be safe for handling, inexpensive and yield solutions of relatively low viscosity in the carrier at working concentration and temperature.

In reversed-phase displacement chromatography, the displacer should be strongly retained on the stationary phase yet soluble in water. Thus in structure it

should have both hydrophobic and hydrophilic moieties. On the basis of earlier scouting experiments we have selected the four substances listed in Table I as possible displacers for the separation of the products of MFBL hydrolysis. They are easily available, bind sufficiently strongly to silica bound hydrocarbonaceous stationary phases, completely miscible with water and are not expected to react with any component of the feed.

TABLE I

RETENTION FACTORS OF THE TWO HYDROLYSIS PRODUCTS, A AND B, OF MFBL AND THE FOUR DISPLACERS UNDER INVESTIGATION AS MEASURED BY LINEAR ELUTION CHROMATOGRAPHY

Column, 250 × 4.6 mm, 5-μm Zorbax ODS; eluent, 15% acetonitrile in 10 m*M* phosphate buffer, pH 2.3; temperature, 22°C.

Eluite	*k'*
Product A	0.10
Product B	0.39
Dipropylene glycol monomethyl ether (DPM)	1.10
Tripropylene glycol monomethyl ether (TPM)	3.40
2-Butoxyethanol (BE)	2.89
2-(2-Butoxyethoxy) ethanol (BEE)	4.58

The basic requirement for these possible displacers to be more strongly retained than any component of the feed may be quickly and inexpensively estimated from elution data. If the isotherms are Langmuirian or nearly Langmuirian, the magnitude of retention factors, which are given by the initial slope of the isotherms in linear elution chromatography, can be expected to follow roughly the same order as the saturation levels of the isotherms. Thus, the compound that is strongest retained in the elution mode can be expected to displace all others in the displacement mode.

All substances in Table I were retained more strongly than products A and B on the octadecyl-silica stationary phase selected for the separation, as also shown in Table I. Further information is gained by measuring the isotherms of the two substances to be separated and of the four potential displacers. Displacement runs were made with the feed mixture at different concentrations of each displacer and the concentrations of each species in the effluent were measured in the fully developed displacement train. The data were used to construct the isotherms of the displacers by using the theory of frontal chromatography[14] and those of the feed components with the aid of the operating line[12]. The isotherms so obtained are depicted in Fig. 5. It can be seen that the isotherms of products A and B are truncated because the length of the column was not sufficient to achieve isotachic conditions beyond a certain concentration limit. It is seen that for all four prospective displacers the isotherms overlie those of products A and B. In principle, separation should not be affected by the choice of displacer solution but should depend only on the choice of the operating line. In practice, however, it may be convenient to choose a displacer which can be used at a low concentration and allows rapid regeneration after the displacement run. Based on such considerations DPM seems to be a suitable displacer for the separation of interest.

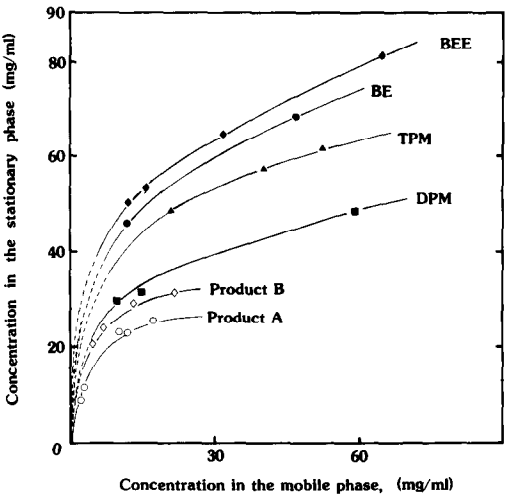


Fig. 5. Isotherms of 2-(2-butoxyethoxy)ethanol (BEE), 2-butoxyethanol (BE), tripropylene glycol monomethyl ether (TPM), dipropylene glycol monomethyl ether (DPM), products A and B of MFBL hydrolysis. Stationary phase: 5 μm Zorbax ODS. Carrier: water. Temperature: 22°C.

Optimum operating line

The operational behavior of all displacers was examined in terms of operating lines, the slope of which determines the speed of separation and the concentration of the emerging products, and thus, for a given feed and flow-rate, the value of the throughput. The slope of the operating line is proportional to the net retention volume of the displacer front, and the same operating line can be obtained with any of the displacers, using the appropriate concentrations determined by their isotherms. In order to find the optimum operating line, the throughputs of pure product A, and product B at 96% and 100% purity, were calculated and plotted against the normalized breakthrough volume for three displacers at different concentrations in Fig. 6. The normalized breakthrough volume is defined as $(V_B - V_0)/V_0$, where V_B is the breakthrough volume of the displacer front and V_0 the dead volume of the column. At low breakthrough volumes of the displacer, the process takes place rapidly but with low recovery because at high product concentrations in the effluent a large proportion of the individual products remains mixed in adjacent zones of the displacement train, with concomitant decrease in the amount of recoverable pure product. On the other hand, upon decreasing the displacer concentration the separation slows down and the final concentrations of the products are reduced. The resulting increase in the volume occupied by the emerging bands allows the recovery of a higher fraction of pure product.

However, the higher recovery is obtained at the price of slowing down the separation process, so that the throughput or production rate is reduced. The interplay of the two opposite effects is illustrated by the plots of experimental results in Fig. 6. In each case there is a critical front velocity of displacer where the throughput is a maximum, as has been pointed out previously[15]. Furthermore the data also illustrate the trade-off between throughput rate and desired purity of the product.

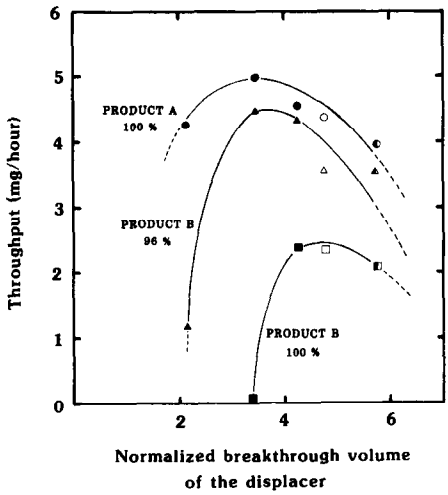


Fig. 6. Graph illustrating plots of throughput *versus* the normalized breakthrough volume of the displacer. Column: 250 × 4.6 mm, 5-μm Zorbax ODS. Carrier: water; flow-rate, 0.1 ml/min. Temperature: 22°C. Feed: 31 mg of the degradation products of MFBL in 1 ml of water. Symbols refer to the displacer used for the experiment: solid symbols, tripropylene glycol monomethyl ether at 40, 21 and 15 mg/ml: open symbols, dipropylene glycol monomethyl ether at 10 mg/ml; half-solid symbol, 2-(2-butoxyethoxy)ethanol at 11.8 mg/ml.

Optimum flow-rate and amount of feed

The interplay of other operating parameters has also to be considered in establishing optimum conditions for the separation process. On the basis of the results discussed above DPM was selected as the best displacer for further experiments to examine the effect of the amount of feed and of the flow-rate on the throughput rate. In view of Figs. 5 and 6 the concentration of DPM was fixed at 10 g/l which facilitates maximum throughput of products A and B at high purities.

Fig. 7 shows the effect of the amount of feed on the throughput rate under otherwise fixed conditions. The results illustrate that with low loading the zones in the displacement train are narrow and consequently the fraction of pure product recovered is also low, as the overlap between zones is relatively high. On the other hand isotachic conditions are not attained when the amount of feed exceeds a certain value, and as a result the separation is incomplete and the recovery is low again. Thus for a given set of conditions and feed composition the optimum amount of feed results in a fully developed displacement train which occupies the column.

Zone overlap in the fully developed displacement train is due to axial dispersion, a well-studied phenomenon in elution chromatography[16]. Since it is a dynamic process its magnitude predominantly depends on the mass transfer resistances and flow maldistribution in the column. This is illustrated in Fig. 8 by the marked effect of the flow-rate on the throughput of the hydrolysis products of MFBL. In the absence of axial dispersion and thus zone overlap, the throughput would be proportional to the flow-rate. However, as seen in Fig. 8 bandspreading causes a decline in production rate at higher mobile phase velocities. Of course relatively slow displacement kinetics at the surface of the stationary phase may also be responsible for zone intermixing.

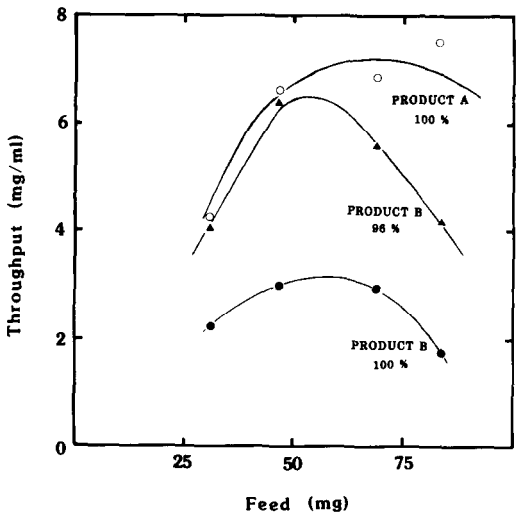


Fig. 7. Graph illustrating plots of throughpout *versus* the amount of feed loaded into the column. Column: 250 × 4.6 mm, 5-μm Zorbax ODS. Carrier: water. Displacer: 10 mg/ml of DPM; flow-rate, 0.1 ml/min. Temperature: 22°C.

Effect of the carrier

In the design of a displacement chromatographic system numerous other operational parameters besides those treated above have also to be considered. In the following some of them will be discussed by examining the results obtained in separating other mixtures.

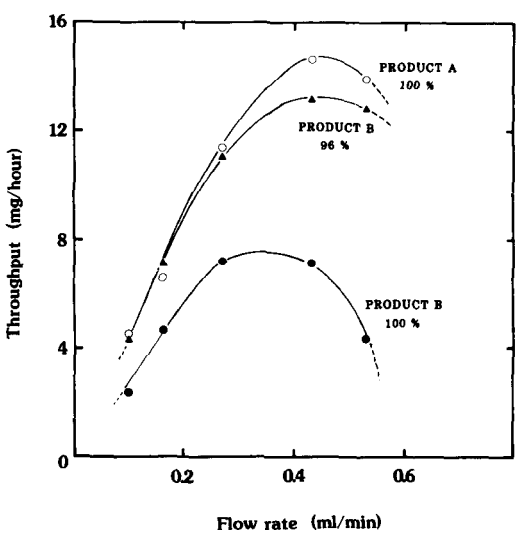


Fig. 8. Graph illustrating plots of throughpout *versus* flow-rate. Feed: 31 mg of the degradation products of MFBL in 1 ml of water. Other conditions as in Fig. 7.

The separation of the two diastereoisomers of BPAP sheds light on the role of the stationary phase and the carrier composition in resolving a mixture of two closely related substances having a low separation factor. In the experiments which were carried out with octadecyl silica columns, *n*-butanol was chosen as the displacer for its strong retention on the stationary phase and other desirable features: electrostatic neutrality at the pH of the carrier, relatively high solubility in water and relatively low vapor pressure and cost.

The carrier in displacement chromatography should be a strong solvent for the feed components and a weak eluent for them on the stationary phase chosen. In reversed-phase chromatography with hydrocarbonaceous silica bonded phases hydro-organic mobile phases are used most widely for elution. However, addition of organic modifier to aqueous carrier lowers the adsorption capacity of such stationary phases and thus the efficiency of the separation by displacement development may be reduced. Nevertheless, in certain cases the gain in feed solubility or other possible benefits may warrant the use of hydro-organic carriers instead of neat aqueous ones. In the case of the BPAP mixture, the solubility of the feed is a critical parameter since the concentration of a saturated solution of BPAP in water is about 10 g/l. Fig. 9 shows the effect of using a water–methanol mixture as the carrier to enhance solubility. The carriers were neat water and water–methanol (90:10, v/v) in the separations illustrated in Fig. 9A and B, respectively. Comparison of the two chromatograms shows that higher product concentrations and faster separation are obtained using the carrier containing methanol. With neat water as the carrier similar results could be obtained at sufficiently high displacer concentration, but at the risk of plugging the column by precipitation of the BPAP components. Thus, within a certain range, the addition of organic modifier to the carrier may affect throughput in the same way as an increase in displacer concentration. The other factors particular to the given chromatographic system have to be considered in the design of operating conditions. It can also be seen in Fig. 9A that both products leave the column at higher concentrations than that in the feed, which was a saturated solution of BPAP. This result demonstrates that individually each of the diastereoisomers has higher solubility in water than when they are present together in BPAP.

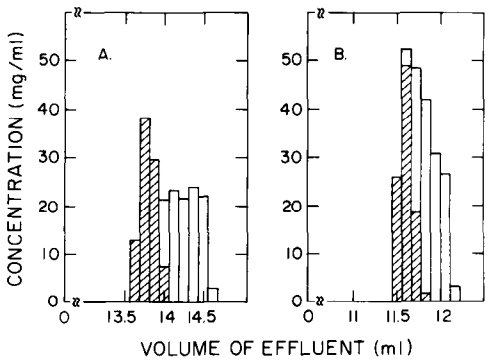


Fig. 9. Effect of composition of carrier on resolution. Column: 400 × 4.6 mm, 5-μm Spherisorb ODS. Carriers: A, water; B, methanol–water (10:90). Displacer: 40 mg/ml of *n*-butanol; flow-rate, 0.5 ml/min. Temperature: 60°C. Fraction volume: 100 μl. Feed: 25 mg BPAP in 2.5 ml. The concentrations of the two diastereomers in the fractions are shown by the hatched and blank bars.

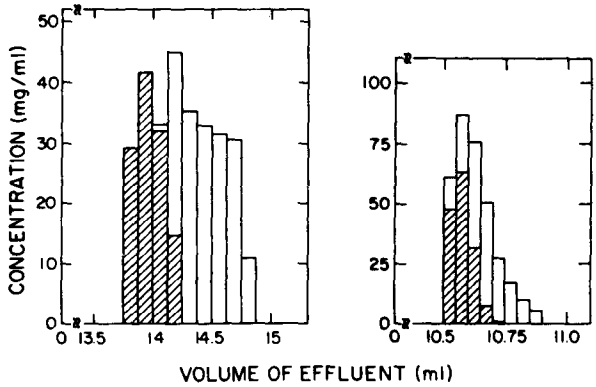


Fig. 10. Effect of stationary phase on the resolution of BPAP diastereoisomers. A, Column: 400 × 4.6 mm, 5-μm Spherisorb ODS. Flow-rate: 0.6 ml/min. Fraction volume: 120 μl. Feed: 30 mg of BPAP in 3.0 ml. B, Column: 250 × 4.6 mm, 10-μm Partisil ODS. Flow-rate: 0.26 ml/min. Fraction volume: 78 μl. Feed: 20 mg of BPAP in 2.0 ml. The displacer, 40 mg/ml of *n*-butanol; the carrier, water and the temperature, 22°C, were the same in the experiments with both systems. The concentrations of the two diastereomers in the fractions are shown by the hatched and blank bars.

Effect of stationary phase

In another set of experiments the effect of stationary phase properties on the separation of BPAP was investigated. Experiments with elution development indicated that alkyl silica offers an efficient and convenient means to bring about the separation. We have found that silica bound octadecyl stationary phases prepared from different supports exhibit marked differences as far as separation efficiency is concerned. Fig. 10A and B show the results of two displacement runs attempted on an octadecyl-Spherisorb and octadecyl-Partisil under similar conditions. Since the octadecyl-Partisil column was shorter than the Spherisorb ODS column, the amount of feed was reduced proportionally on the basis of theoretical considerations[2]. As seen in Fig. 10B no complete displacement occurred in the Partisil ODS column with the same displacer solution and the separation of the components was extremely poor. In fact the BPAP components eluted behind the displacer front, thus *n*-butanol was not a displacer on the Partisil based stationary phase, whereas it displaced effectively the sample components on the Spherisorb based octadecyl silica. Partisil has greater specific surface area and wider pore distribution than Spherisorb and the carbon contents of the respective octadecyl derivatives were 10.9 and 13.5%. In view of this the Partisil based octadecyl-silica is believed to have a smaller surface coverage than the stationary phase made from Spherisorb and a higher concentration of silanol groups, to which the components of the feed bind strongly. Under such circumstances their isotherms may not be overlaid by that of *n*-butanol which therefore cannot displace the BPAP components from the surface of this stationary phase.

Experiments were carried out in order to improve the separation on the Partisil ODS column by using displacers having a stronger interaction with the silanol groups than *n*-butanol. 2-(2-Butoxyethoxy)ethanol failed to displace BPAP and the components were eluted as with *n*-butanol. Tetrabutylammonium bromide, which was used previously in displacing peptides[13], bound to BPAP even at low pH and prevented the separation. Carrier displacement[15] was also attempted by using Carbowax

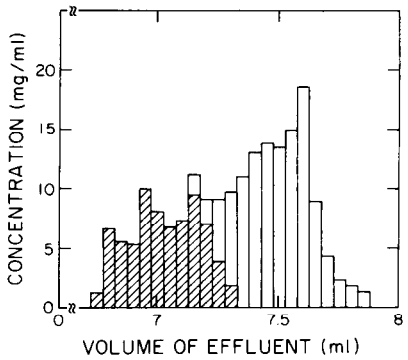


Fig. 11. Separation of BPAP components. Column: 250 × 4.6 mm, 5-μm Partisil ODS. Carrier: water. Displacer: 100 mg/ml Carbowax PEG-1000; flow-rate, 0.25 ml/min. Fraction volume: 25 μl. Temperature: 22°C. Feed: 15 mg of BPAP in 1.5 ml. The concentrations of the two diastereomers in the fractions are shown by the hatched and blank bars.

PEG-600 as spacer between the two BPAP disasteromers, but it was ineffectual in this mode. Carbowax PEG-4000, which has higher molecular weight than the polyethylene glycols employed before, was also evaluated but it was very strongly retained by Partisil ODS so that the BPAP eluted ahead of it, and its high viscosity prevented its use at concentrations higher than 65 g/l. However, Carbowax PEG-1000, having intermediate molecular weight, displaced the BPAP mixture at a concentration of 65 g/l, as shown on the chromatogram in Fig. 11. It is seen that none of the bands are rectangular and that the concentration of the components in the zones are greater than in the feed as discussed before. Comparison of Fig. 11 to Fig. 10A shows that when using Carbowax PEG-1000 as the displacer with a Partisil ODS column the separation efficiency is very similar to that obtained on Spherisorb ODS with *n*-butanol. This demonstrates that in optimizing the separation of a given mixture the stationary phase and displacer have to be considered together.

The particle size of the packing material is also a critical parameter, since column efficiency increases as in elution chromatography with decreasing the particle size. The effect of particle size on the separation is illustrated in Fig. 12 where a 250

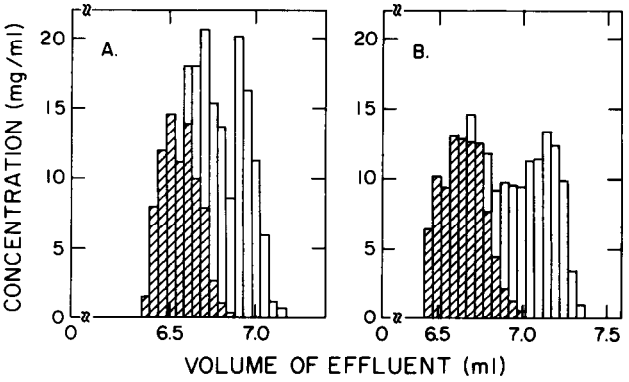


Fig. 12. Separation of BPAP components. Column: 250 × 4.6 mm, 10-μm Partisil ODS. Carrier: water. Displacer: 65 mg/ml Carbowax PEG-400; flow-rate, 0.3 (A), 0.15 ml/min (B). Temperature: 22°C. Fraction volume: 60 μl. Feed: 10 mg/min of BPAP in 1 ml. The concentrations of the two diastereomers in the fractions are shown by the hatched and blank bars.

× 4.6 mm, 10-μm Partisil ODS column was used. The flow-rate was decreased in an attempt to relax the effect of mass transfer and kinetic resistances in the displacement process, since both phenomena play an important role in determining the efficacy of the separation with columns using large particle size. An attempt was made to use a 250 × 4.6 mm, 40-μm Partisil ODS column, but technical limitations on the pump employed made it impossible to reduce the flow-rate to a level at which this column may have yielded good separation. The use of small particles (if possible) is advantageous because in addition to increased yield of pure product, relatively high flow-rates can be used, with the result of increased throughput. On the other hand theoretical arguments predict a minimum size of the particles beyond which no advantage is gained by increasing the flow-rate, because the efficiency of the column is effectively controlled by the slowness of displacement kinetics on the surface of the stationary phase.

Effect of column diameter

The separation of ten polyethylene glycol oligomers present in Carbowax PEG-400 is used to illustrate certain aspects of scaling-up the process from analytical columns to preparative columns having five times greater inner diameter. Fig. 13 shows the separation of the components of Carbowax PEG-400 by using a 250 × 9.4 mm octadecyl silica column. The chromatographic results illustrate the promise of displacement development for the separations of oligomers, a problem frequently encountered in biological applications. The number of ethoxy units in each of the components which form the zones on the chromatogram depicted in Fig. 13 was tentatively established by high-performance liquid chromatography (HPLC) analysis. The feed was 400 mg of Carbowax PEG-400 dissolved in 1.0 ml of water, and most of the components were obtained not only in pure form but also at relatively high concentrations. It is seen that the feed components having 1, 2, 9 and 10 ethoxy residues are present in relatively small amounts, and so form very narrow bands in the displacement train. Consequently the recovery of these components is lower than

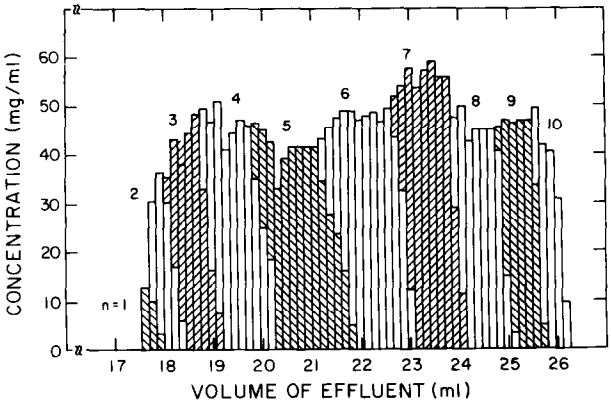


Fig. 13. Separation of Carbowax PEG-400. Column: 250 × 9.4 mm, 10-μm Partisil ODS. Carrier: water. Displacer: 65 mg/ml of tetrabutylammonium bromide; flow-rate, 0.8 ml/min. Temperature: 22°C. Fraction volume: 160 μl. Feed: 400 mg Carbowax PEG-400 in 1 ml. The concentrations of the individual components of PEG-400 in the fractions are shown by the hatched and blank bars. The number of ethylene oxide residues is given by *n*.

those present at higher concentrations in the feed and forming much wider bands. The advantages of displacement chromatography as a premier process for multicomponent separation at high capacity and efficiency is handsomely revealed in Fig. 13. A complex mixture is separated without the excessive tailing and dilution of the later-eluting peaks encountered at high column load in isocratic elution chromatography where the efficiency needed to resolve such closely related compounds would be hard to attain for a preparative scale separation. Nevertheless Fig. 13 shows that there is significant tailing of the individual bands. This is likely to be due to a secondary equilibrium process involving conformation change of the polyethylene glycols as has been shown to occur in elution chromatography[17]. Most likely a change in column temperature and/or use of hydro-organic carrier would have been needed to do away with tailing due to such phenomena.

An attempt was made to scale-up this separation by using a wider column having 22 mm inner diameter. In the elution mode the efficiencies of these columns were comparable to analytical columns in terms of plate height for benzene and naphthalene but tailing was much more pronounced with the wide column, according to the manufacturer. Tailing and the concomitantly low recovery was probably due to a poor distribution of the feed at the column entrance, a problem which is encountered also in scaling-up elution chromatography[18]. In our experiments no attempt was made to optimize the configuration of the column entrance in order to alleviate untoward entrance effects.

A simple way of improving the efficiency of displacement chromatography with wide columns is to connect to the outlet a narrow bore column which serves to sharpen the boundaries and thus enhances recovery[19]. Recoveries and throughputs of pure species, shown in Table II for the separations described above, indeed confirm

TABLE II

YIELD AND THROUGHPUT OBTAINED IN THE SEPARATION OF CARBOWAX PEG-400 SEPARATION WITH 10-μm PARTISIL ODS COLUMNS USING DIFFERENT INNER DIAMETERS

The length of each column was 250 mm. Carrier: water. Displacer: 65 mg/ml tetrabutylammonium bromide. Temperature 22°C. Different flow-rates and sample feeds were used according to the inner diameter of the column: 0.08 mg/ml and 400 mg for 9.4 mm I.D., 4.4 ml/min and 2200 mg for 22 mm I.D., 1 ml/min and 2200 mg for the two-columns system.

Number of ethoxy residues in the component	*Recovery (%)*			*Throughput (mg/h)*		
	9.4 mm I.D.	*22.0 mm I.D.*	*22.0 + 4.6 mm I.D.*	*9.4 mm I.D.*	*22.0 mm I.D.*	*22.0 + 4.6 mm I.D.*
1	49	28	74	3.4	8.4	9
2	31	52	61	8.6	62.5	31
3	44	51	74	24.4	122.7	74
4	51	55	70	45.9	215	114
5	65	61	67	67.5	275	126
6	52	63	77	61.2	322	164
7	75	55	59	88.3	281	126
8	61	17	41	46.5	56	56
9	44	0	37	24.4	0	37
10	30	0	27	10	0	17

the recovery is increased by using such a two-column system, though the throughput of the products present in largest amounts in the feed is decreased by the lower flow-rate necessary to relax kinetic resistances to equilibration in the narrow column as well as to keep the column inlet pressure constant. Thus with recovery as the criterion narrow columns appear to be superior, whereas the much greater loading capacity of the wider bore columns resulted in higher throughput despite the fact that entrance conditions were not optimized.

CONCLUSIONS

Displacement chromatography with columns and equipment used in HPLC has proved itself to be an efficient technique for the separation of complex mixtures of closely related compounds such as those frequently encountered in biological systems. Although displacement development is still perceived as a more complex process than elution from the theoretical and operational points of view recent advances give more and more insight into the underlying physico-chemical phenomena and facilitates the solution of problems inherent to the practice of displacement chromatography.

In optimization of displacement development numerous factors must be considered. Upon selecting a suitable column the most important is the nature and the concentration of the displacer which determine the speed and the efficiency of the separation. Various displacers can be used to separate the same mixture, and it was possible to define for a given reversed-phase column an optimum slope in term of throughput for the operating line, as well as an optimum feed load and flow-rate. Interplay of other factors like the composition of the carrier or the characteristics of the stationary phase have been illustrated.

Scaling up of a separation performed on analytical column to preparative columns of wider inner diameter still requires further technical improvements to eliminate the loss of efficiency critical in displacement chromatography where the components are separated into adjacent bands. However, the high concentrations of the products in the effluent and the possibility of introducing much higher amounts of feed than in the elution mode in this non-linear mode of chromatography make this process one of the techniques *par excellence* for preparative separations.

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